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Effect of suppressive DNA on CpG-induced immune activation.

Yamada H, Gursel I, Takeshita F, Conover J, Ishii KJ, Gursel M, Takeshita S, Klinman DM.

Section of Retroviral Immunology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892, USA.

Bacterial DNA and synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs stimulate a strong innate immune response. This stimulation can be abrogated by either removing the CpG DNA or adding inhibitory/suppressive motifs. Suppression is dominant over stimulation and is specific for CpG-induced immune responses (having no effect on LPS- or Con A-induced activation). Individual cells noncompetitively internalize both stimulatory and suppressive ODN. Studies using ODN composed of both stimulatory and suppressive motifs indicate that sequence recognition proceeds in a 5'-->3' direction, and that a 5' motif can block recognition of immediately 3' sequences. These findings contribute to our understanding of the immunomodulatory activity of DNA-based products and the rules that govern immune recognition of stimulatory and suppressive motifs.

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Brief Communication

Ability of Oligonucleotides with Certain Palindromes to Induce Interferon Production and Augment Natural Killer Cell Activity Is Associated with Their Base Length

TOSHIKO YAMAMOTO,[1] SABURO YAMAMOTO,[1] TETSURO KATAOKA,[1] and TOHRU TOKUNAGA[2]

ABSTRACT

A synthetic 30-mer single-stranded oligodeoxyribonucleotide with a hexamer palindrome, AACGTT, induced IFN production and augmented NK activity in murine splenocytes. This effect does not appear to result from an antisense mechanism but rather is due to the palindrome. To clarify the required minimal size of the nucleotide, 10 kinds of 12- to 30-mer nucleotides were examined. Immunostimulatory activity of oligonucleotides 18 bases or more in length was observed and was proportional to the base length, with a maximum at 22–30 bases. On the other hand, the oligonucleotides 16 bases or less in length were not active even if they possessed the palindromic sequence. These results indicate that the immunostimulatory activity of oligonucleotides with certain palindromic sequences requires an oligonucleotide at least 18 bases long.

INTRODUCTION

A DNA-RICH FRACTION extracted and purified from *Mycobacterium bovis* BCG, MY-1, showed strong antitumor activity against various syngeneic tumors in mouse and guinea pig (Tokunaga et al., 1984; Shimada et al., 1985). This fraction augmented *in vitro* natural killer (NK) cell activity of murine spleen cells and human peripheral blood (Shimada et al., 1986; Mashiba et al., 1988; Yamamoto et al., 1988). It also induced production of interferon α (IFN-α) with small amounts of IFN-β and -γ and macrophage-activating factor (Mashiba et al., 1988; Yamamoto et al., 1988). These activities were destroyed by treatment of MY-1 with DNase, but not with RNase (Yamamoto et al., 1988). Thus the DNA component of MY-1 was considered to be responsible for the biological activities.

Previously we synthesized a variety of single-stranded oligodeoxyribonucleotides (oligoDNAs), sequences of which were chosen from cDNA encoding the 64-kDa heat shock protein (Ag A) of BCG (Thole et al., 1987), to assess the biological activity (Tokunaga et al., 1992; Yamamoto et al., 1992). One 45-mer oligoDNA (nucleotides 813–857 of the cDNA of Ag A), designated BCG-A4, augmented NK activity of murine spleen cells *in vitro*, whereas another 45-mer oligoDNA (nucleotides 694–738 of the same cDNA), designated BCG-A2, did not. Moreover BCG-A4a, which was a 30-mer oligoDNA with the 5′-end sequence of BCG-A4 and which possessed one palindromic sequence (GACGTC), was also active whereas BCG-A4b, which was a 30-mer with the 3′-end sequence of BCG-A4 and which possessed no palindromic sequence, was inactive. Fifteen-mer nucleotide fragments of BCG-A4, even if some of them possessed the palindromic sequence, had no activity (Yamamoto et al., 1992). These findings suggested that a unique palindromic sequence and some molecular size of synthetic oligoDNA was required to induce the biological activity. Furthermore, the hexamer palindromic sequence GACGTC in BCG-A4a (the sequence of which is 5′ ACCGATGACGTCGCCGGTGACGGCACCACG 3′) was replaced with 64 different palindromic base combinations. Of the 64 palindromes, only 9 palindromic sequences supported the biological activity of the 30-mer DNAs (Kuramoto et al., 1992). This finding suggested that the 9 potent oligoDNAs did not act by antisense mechanisms, and it also indicated that a variety of steric

[1]Department of Bacterial and Blood Products, [2]National Institute of Health, Gakuen, 4-7-1, Musashi-Murayama-shi, Tokyo 208, Japan.

structures among the 64 kinds of hexamer palindromes, or some unique structure(s) of oligoDNA(s), might be responsible for the biological activity. On the basis of these results, we selected two BCG-A4a analogs designated AAC-30 and ACC-30 (Table 1) for further study of the steric structures and biological activity f oligoDNAs. AAC-30, having the AACGTT palindrome, is different by only two bases from ACC-30, having the ACCGGT palindrome; but the former induced IFN production and NK cell augmentation whereas the latter did not (Kuramoto et al., 1992; Yamamoto et al., 1992). In this study, we synthesized 13 kinds of oligoDNAs of various base lengths and determined the minimal molecular size of oligoDNA required for induction f IFN production and augmentation of NK activity.

Oligodeoxyribonucleotides were synthesized with an automatic synthesizer (Gene Assembler Plus; Pharmacia-LKB, Uppsala, Sweden) by the standard phosphoramidite method (Agrawal et al., 1989). After purification by gel filtration, dialysis, and ethanol precipitation, the oligoDNAs were lyophilized. Their concentration was determined by absorbance at 260 nm, after being resolved with saline. Purity of the oligoDNAs was confirmed by high-performance liquid chromatography (HPLC) and polyacrylamide gel electrophoresis (PAGE). MY-1 was prepared as described previously (Tokunaga et al., 1984). Normal BALB/c female mice (8–12 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). Mouse lymphoma YAC-1 cells and fibroblast L929 cells were maintained in tissue culture using RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Assay for IFN level and NK activity was done as described previously (Yamamoto et al., 1992). Briefly, mouse spleen cells at a concentration of 1×10^7 cells/ml were incubated with 5×10^{-9} M (or 50 μg/ml) oligoDNA for 20 hr at 37°C. The cells were used as NK cells and the levels of IFN in the culture supernatants were measured. Natural killer cell activity was calculated as percent lysis, using ^{51}Cr-labeled YAC-1 cells as target. One million cultured spleen cells were mixed with 1×10^4 target cells in a final volume of 0.2 ml and distributed to the wells of a 96-well U-bottomed plate, and the cell mixture were cultured for 4 hr at 37°C. After incubation, the supernatants were collected and the radioactivity released from the target cells was measured by a standard γ scintillation counter. The percentage of target cell lysis was calculated by the following formula: [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] × 100. The levels of IFN in the culture supernatants were measured in terms of the ability to inhibit cytopathic effects of vesicular stomatitis virus on mouse L929 fibroblasts. L929 cells (5×10^4) were seeded in a 96-well flat-bottomed plate in a volume of 50 μl. Twenty-four hours later, when the cells had become confluent, the wells were filled with additional 50-μl aliquots of twofold serial dilutions of the culture supernatants or the standard IFN, the titer of which was equivalent to the National Institutes of Health (NIH) reference IFN. After incubation for further 24 hr, the cultured L929 cells were treated with the virus in a 100-μl dilution containing 10 $TCID_{50}$ (50% tissue culture infectious dose). The cytopathic effect was examined microscopically after 40 hr of incubation. Antiviral units were expressed in international units (IU) per milliliter, which was calculated from the highest dilution for 50% inhibition of the cytopathic effect. Oligonucleotides had no direct effect on L929 cells. For preparation of AAC oligoDNAs of various base lengths, the extrapalindromic sequence of AAC-30 was trimmed stepwise. Trimming two nucleotides from the 3′ end of the AAC-30 sequence resulted in AAC-28. By repeating this procedure, AAC-26, AAC-24, AAC-22, AAC-20, AAC-18, AAC-16, AAC-14, and AAC-12 were selected. In these procedures, only extrapalindromic sequences were trimmed and a hexamer palindrome of AACGTT was maintained. Ten kinds of oligoDNA analogs having a hexamer palindromic sequence AACGTT were synthesized. Similarly, trimming of ACC-30 resulted in ACC-22 and ACC-20 (Table 1).

The activities of these oligoDNAs in inducing IFN production and augmenting NK cell activity are shown in Table 2. When murine spleen cells were cocultured with 5×10^{-9} M (or 50 μg/ml) ACC-30 analogs, seven oligoDNAs (AAC-30, AAC-28, AAC-26, AAC-24, AAC-22, AAC-20, and AAC-18) showed activity. On the other hand, AAC-14 induced slight NK activity but not IFN production, and AAC-12 showed no activity. In the case of AAC-16, the concentration was critical; 50

TABLE 1. SEQUENCE OF AAC AND ACC oligoDNAs OF VARIOUS BASE LENGTHS

Name	*Base sequence (5′ → 3′)*[a]				
AAC-30	ACCGAT	AACGTT	GCCGGT	GACGGC	ACCACG
AAC-29	ACCGAT	AACGTT	GCCGGT	GACGGC	ACCA
AAC-26	ACCGAT	AACGTT	GCCGGT	GACGGC	AC
AAC-24	ACCGAT	AACGTT	GCCGGT	GACGGC	
AAC-22	ACCGAT	AACGTT	GCCGGT	GACG	
AAC-20	ACCGAT	AACGTT	GCCGGT	GA	
AAC-18	ACCGAT	AACGTT	GCCGGT		
AAC-16	ACCGAT	AACGTT	GCCG		
AAC-14	ACCGAT	AACGTT	GC		
AAC-12	ACCGAT	AACGTT			
ACC-30	ACCGAT	ACCGGT	GCCGGT	GACGGC	ACCACG
ACC-22	ACCGAT	ACCGGT	GCCGGT	GACG	
ACC-20	ACCGAT	ACCGGT	GCCGGT	GA	

[a]Underlined sequences are palindromic.

TABLE 2. PRODUCTION OF INTERFERON AND AUGMENTATION OF NATURAL KILLER CELL ACTIVITY BY MURINE SPLEEN CELLS STIMULATED WITH AAC AND ACC oligoDNAs

	IFN (IU/ml)		*NK activity*[b] *(% lysis ± SD)*
OligoDNA[a]	*5 × 10⁻⁹ M*	*50 μg/ml*	*(50 μg/ml)*
AAC-30	128	128	25.8 ± 1.2
AAC-28	128	128	28.2 ± 1.0
AAC-26	128	128	29.6 ± 1.8
AAC-24	128	128	29.8 ± 1.7
AAC-22	128	128	30.0 ± 0.8
AAC-20	64	64	26.4 ± 0.5
AAC-18	8	32	18.3 ± 0.6
AAC-16	<4	4	13.9 ± 0.2
AAC-14	<4	<4	13.0 ± 0.5
AAC-12	<4	<4	11.6 ± 0.4
AAC-30	<4	<4	11.7 ± 1.3
AAC-22	<4	<4	11.4 ± 0.8
AAC-20	<4	<4	11.0 ± 0.6
MY-1	128	128	25.5 ± 1.0
Medium	<4	<4	10.4 ± 0.4

[a]The sequences of the oligoDNAs used are listed in Table 1.
[b]Spontaneous counts per minute was 1139 and total counts per minute was 9561.

μg/ml induced IFN production but a 5×10^{-9} M concentration, which is equal to 27 μg/ml, did not. The difference in concentration between 50 μg/ml and 5×10^{-9} M AAC-18 might have caused the difference in IFN level. None of the oligoDNAs having another palindromic sequence of ACCGGT (ACC-30, ACC-22, and ACC-20) showed the activity. These results suggested that the level of IFN was influenced by the concentration of intracellular potent oligoDNA, and it was confirmed by our experimental results of lipofection of AAC-22. When AAC-22 was encapsulated with liposome and transfected into murine spleen cells, the same level of IFN induced by 50 μg of naked AAC-22 per milliliter was induced by a low concentration of 0.016 μg/ml; lipofection was 3000-fold more efficient than coculture. Spleen cells transfected with liposome-encapsulated ACC-22 showed only a slight enhancement of IFN induction. Furthermore, when binding of AAC-22 to spleen cells was compared with that of ACC-22, no difference was observed between them (Yamamoto et al., 1993). These results strongly suggest that IFN production is triggered by entry of AAC-22 inside spleen cells but not by binding to cell surface receptors.

The NK activity augmented by AAC oligoDNAs was elevated with the level of IFN. But the NK activity augmented by AAC-30 was weaker than that of AAC-28, although the level of IFN was same. And in AAC-14 a slight augmentation of NK activity was recognized without IFN. Taken together with the indication from our previous study that IFN-α/β is responsible for the augmentation of NK activity by AAC-30 (previously designated A4a-AAC) (Yamamoto et al., 1992), the results shown in Table 2 suggest that another cytokine induced by AAC oligoDNAs may play a minor role in the augmentation of NK activity.

Eighteen- to 30-mer AAC oligoDNAs induced IFN in murine splenocytes *in vitro*, whereas AAC-12 and AAC-14 did not, even though they had the same palindromic sequence. These results indicate that the minimal base length of immunostimulatory oligoDNAs having a potent palindromic sequence (AACGTT) is 18, of which the molecular weight is about 6000. This molecular size may be required to maintain some unique steric structure to express the activity, and the structure may be more stable in AAC-20. The stability of the structure may reach an equilibrium state in AAC-22. BCG-A4a has a hexamer palindromic sequence (GACGTC), and this sequence is recognized by the restriction enzyme *Pst*I. A hairpin structure of CTGCAG in the synthesized 12-mer double-stranded DNA (dsDNA), having the sequence 5′ ACCCTGCAGGGT 3′, was observed by nuclear magnetic resonance (NMR) spectroscopy during the dissociation process from the double strand to single strand (H. Nakanishi [National Institute of Bioscience and Technology, Tsukuba, Japan], personal communication, 1993). This information supports our speculation that the potent palindromes may have some unique steric structure(s) to express the activity.

Although the reason why the difference in immunostimulatory activity between AAC oligoDNAs and ACC oligoDNAs was seen is still obscure, the studies on the steric structures of these oligoDNAs and expected regulatory protein(s) that bind AAC oligoDNAs will resolve the question. Further study of the steric structures of AAC-22 and ACC-22 by NMR spectroscopy is in progress.

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Address reprint requests to:
Toshiko Yamamoto
Department of Bacterial and Blood Products
National Institute of Health
Gakuen, 4-7-1, Musashi-Murayama-shi
Tokyo 208, Japan

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Immunostimulatory DNA Sequences Inhibit IL-5, Eosinophilic Inflammation, and Airway Hyperresponsiveness in Mice[1]

David Broide,[2]* Jurgan Schwarze,† Helen Tighe,‡ Tim Gifford,* Minh-Duc Nguyen,‡ Siamak Malek,‡ John Van Uden,§ Elena Martin-Orozco,§ Erwin W. Gelfand,† and Eyal Raz*‡§

We have used a mouse model of allergen-induced airway hyperresponsiveness to demonstrate that immunostimulatory DNA sequences (ISS) containing a CpG DNA motif significantly inhibit airway eosinophilia and reduce responsiveness to inhaled methacholine. ISS not only inhibited eosinophilia of the airway (by 93%) and lung parenchyma (91%), but also significantly inhibited blood eosinophilia (86%), suggesting that ISS was exerting a significant effect on the bone marrow production of eosinophils. The inhibition of the bone marrow production of eosinophils by 58% was associated with a significant inhibition of T cell-derived cytokine generation (IL-5, granulocyte-macrophage CSF, and IL-3). ISS exerted this inhibitory effect on T cell cytokine production indirectly by stimulating monocytes/macrophages and NK cells to generate IL-12 and IFNs. The onset of the ISS effect on reducing the number of tissue eosinophils was both immediate (within 1 day of administration) and sustained (lasted 6 days), and was not due to ISS directly inducing eosinophil apoptosis. ISS was effective in inhibiting eosinophilic airway inflammation when administered either systemically (i.p.), or mucosally (i.e., intranasally or intratracheally). Interestingly, a single dose of ISS inhibited airway eosinophilia as effectively as daily injections of corticosteroids for 7 days. Moreover, while both ISS and corticosteroids inhibited IL-5 generation, only ISS was able to induce allergen-specific IFN-γ production and redirect the immune system toward a Th1 response. Thus, systemic or mucosal administration of ISS before allergen exposure could provide a novel form of active immunotherapy in allergic diseases. *The Journal of Immunology,* **1998, 161: 7054–7062.**

Allergic asthma is characterized by cellular infiltration of the airways with eosinophils and T lymphocytes expressing a Th2 profile of cytokines (1, 2). This characteristic inflammatory response is evident both in bronchial biopsies obtained from asthmatic patients as well as in mouse models of altered airway responsiveness. Following allergen inhalation in sensitized subjects or animals, Th2 cells release a particular set of cytokines (i.e., IL-5, GM-CSF,[3] and IL-3) that promote airway eosinophilia by several different mechanisms, including induction of eosinophil proliferation in the bone marrow, promotion of the release of eosinophils from the bone marrow, and inhibition of eosinophil apoptosis (3, 4). In addition to promoting airway eosinophilia, these Th2 cytokines prime and activate eosinophils to release proinflammatory cytoplasmic granule products, lipid mediators, and cytokines that are thought to contribute to the tissue damage, remodeling, and hyperresponsiveness of the asthmatic airways (1). Antiinflammatory medications such as corticosteroids are standard therapy for asthma, but have limitations in that they may not be disease modifying (asthma recurs when the corticosteroids are discontinued). In addition, corticosteroids, even when delivered by the inhalation route, are associated with the potential for significant side effects, including cataracts, growth retardation, and osteoporosis. Therefore, there is a need to develop safe and effective alternative therapies to corticosteroids to inhibit the critical events (e.g., Th2 cell activation) that initiate and perpetuate eosinophilic inflammation in the airways.

More than a decade ago, Tokunaga and coworkers discovered that DNA purified from mycobacteria induced the release of IFNs from splenocytes (5). Fractionation of the mycobacterial DNA led to the isolation of several short DNA sequences (containing CpG dinucleotide cores) that mediated the immunostimulatory activity (6). Subsequent experiments have shown that oligodeoxynucleotides (ODN) containing ISS (ISS-ODN) induce the release of several additional cytokines, including IFN-α,β (5, 6) IL-6 (7), IL-12 (8, 9), and IL-18 (10) primarily from monocytes (6, 10, 11), and IFN-γ from NK cells (6). The immune response triggered by ISS is similar to the innate immune response evoked by intracellular pathogens (10), triggering the release of cytokines that bias the immune response toward development of an Ag-specific Th1 effector and memory response (10). Cellular activation by ISS DNA such as CpG is not mediated through binding to a cell surface receptor, but requires cellular uptake by adsorptive endocytosis (12). Studies suggest that CpG DNA, taken up by B cells and monocytes by adsorptive endocytosis, is acidified in an intracellular endosomal compartment (12). Endosomal acidification of DNA is coupled to the rapid generation of reactive oxygen species that leads to nuclear factor-κB activation and subsequent cytokine expression (12).

In this study, we have used a mouse model of eosinophilic airway inflammation to investigate whether ISS could inhibit both the generation of Th2 cytokines important to eosinophil proliferation

*Department of Medicine, and §The Sam and Rose Stein Institute for Research on Aging, University of California at San Diego, La Jolla, CA 92093; ‡Dynavax Technologies Corporation, San Diego, CA 92121; and †Department of Pediatrics, National Jewish Medical and Research Center, Denver, CO 80206

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[2] Address correspondence and reprint requests to Dr. D. Broide, Department of Medicine, University of California San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0635.

[3] Abbreviations used in this paper: GM-CSF, granulocyte-macrophage CSF; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; DAB, diaminobenzidine; i.n., intranasal; i.t., intratracheal; MCh, methacholine; ISS-ODN, immunostimulatory sequence-oligodeoxynucleotide; M-ODN, mutated-oligodeoxynucleotide.

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and survival (IL-5, GM-CSF, IL-3), as well as the subsequent airway hyperreactivity in response to methacholine (MCh) challenge. The data presented in this study indicate that administration of ISS-ODN inhibits both airway hyperresponsiveness and airway eosinophilia by exerting a significant inhibitory effect on the generation of eosinophil-active cytokines (IL-5, GM-CSF, and IL-3) as well as the subsequent bone marrow production of eosinophils. Moreover, while both ISS and corticosteroids inhibited IL-5 generation, only ISS was able to induce IFN-γ, a cytokine that importantly biases the immune system to generate a Th1 (and not Th2) response to subsequently encountered allergens.

Materials and Methods

Oligonucleotides

Endotoxin-free (<1 ng/mg DNA) phosphorothioate ISS-ODN (5′-TGACTGTGAACGTTCGAGATGA-3′) or phosphorothioate M-ODN (5′-TGACTGTGAAGGTTAGAGATGA-3′) (Trilink, San Diego, CA), as previously described (10), were used in the in vivo and in vitro experiments described below.

Animals

Female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were used when they reached 8–10 wk of age. All animal experimental protocols were approved by the University of California, San Diego, and the National Jewish Medical Research Center Animal Subjects Committees.

Determination of airway responsiveness to MCh in vivo

In experiments performed at the National Jewish Medical and Research Center, BALB/c mice were sensitized by the i.p. injection of OVA/alum on days 1 and 10, and subsequently received nebulized 1% OVA on days 22, 23, and 24. Airway responsiveness was assessed on day 26, 48 h after the final OVA inhalation, using a single chamber whole body plethysmograph obtained from Buxco (Troy, NY), as previously described (13). In this system, an unrestrained, spontaneously breathing mouse is placed into the main chamber of the plethysmograph, and pressure differences between this chamber and a reference chamber are recorded. The resulting box pressure signal is caused by volume and resultant pressure changes during the respiratory cycle of the mouse. A low pass filter in the wall of the main chamber allows thermal compensation. From these box pressure signals, the phases of the respiratory cycle, tidal volumes, and the enhanced pause (Penh) can be calculated. Penh is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and of the timing of expiration. It correlates closely with pulmonary resistance measured by conventional two-chamber plethysmography in ventilated mice (13). Penh was used to monitor airway responsiveness in this study. In the plethysmograph, mice were exposed for 3 min to nebulized PBS and subsequently to increasing concentrations of nebulized MCh (Sigma, St. Louis, MO) in PBS using an Aerosonic ultrasonic nebulizer (DeVilbiss). After each nebulization, recordings were taken for 3 min. The Penh values measured during each 3-min sequence were averaged and are expressed for each MCh concentration as the percentage of baseline Penh values following PBS exposure (13). To determine the effect of ISS on airway responsiveness in OVA-sensitized mice, 50 μg of either ISS-ODN or M-ODN was injected i.p. 24 h before each of three OVA inhalation challenges, and airway responsiveness was determined (5 days after the first dose of ISS). The number of bronchoalveolar lavage fluid (BALF) eosinophils was assessed in parallel.

Induction of pulmonary allergic eosinophilic inflammation

To investigate whether ISS inhibits airway eosinophilia, the OVA sensitization and challenge protocol of Corry et al. (14) were used. This protocol (14) differs in the number and route of OVA injections and inhalations compared with the protocol used to assess airway hyperresponsiveness. In these studies, mice were immunized s.c. on days 0, 7, 14, and 21 with 25 μg of OVA (OVA, grade V; Sigma) adsorbed to 1 mg of alum (Aldrich) in 200 μl normal saline. The OVA inhalation challenge (days 26 and 31) consisted of three 30-min inhalations (separated by 30-min rest intervals) of OVA at a concentration of 10 mg/ml in an inhalation chamber. The nebulizer (DeVILBISS UltraNeb-99; Sunrise Medical, Sommerset, PA) was set up to aerosolize 80–100 ml of protein solution in the 30-min inhalation time period. The outflow of the inhalation chamber was attached to a vacuum line and adjusted to a minimal suction rate (enough to prevent excess condensation from occurring in the chamber). Mice were sacrificed, and BALF, lungs, peripheral blood, and bone marrow were analyzed 24 h after the second OVA inhalation.

Therapeutic intervention with ISS-ODN or corticosteroids

The therapeutic intervention protocol is summarized in Fig. 2. ISS-ODN or M-ODN was injected i.p. (100 μg in 100 μl of sterile, endotoxin-free PBS) or instilled intranasally (i.n.) (50 μg of ODN/naris, in 20 μl PBS, under metofane anesthesia). The ODNs were administered either twice (on days 25 and 30, 1 day before each OVA inhalation challenge) or once (on day 25, 1 day before the first OVA inhalation challenge; or once on day 30, 1 day before the second OVA inhalation; or on day 31, 30 min before OVA inhalation). Intratracheal (i.t.) administration of ISS-ODN or M-ODN (100 μg of ODN in 50 μl PBS, under metofane anesthesia) was performed twice on days 25 and 30, 1 day before each OVA inhalation challenge.

Dexamethasone (Sigma), 5 mg/kg in 100 μl of PBS, was injected s.c. either twice (days 25 and 30, 18 h before each OVA inhalation) or daily for 7 days from day 25 through day 31.

Eosinophil counts

Twenty-four hours after the second OVA inhalation challenge (day 32), mice were sacrificed by cervical dislocation, and eosinophil counts of various tissues were performed.

Lung. Lung tissues embedded in OCT in 10 × 50 × 50-mm tissue wells were cryosectioned at 10 μ and acetone fixed onto poly(L-lysine)-coated slides. Total eosinophil numbers were enumerated by detection of eosinophil peroxidase using DAB staining and microscopic examination, as described in this laboratory (15). A key code was established for mice groups, and color-coded slides were labeled to designate mouse numbers within groups (i.e., 1–4). Slides were incubated at room temperature for 1 min in the presence of cyanide buffer (10 mM potassium cyanide, pH 6), rinsed in PBS, and incubated for 10 min with the peroxidase substrate DAB (Vector Lab, Burlingame, CA). Slides were subsequently washed in PBS, counterstained with hematoxylin, air dried, and examined by light microscopy (×40 magnification) by a "blinded" examiner. Five random fields were selected and eosinophils were counted (cells staining brown) to determine total eosinophil number per microscope field.

BALF. The sacrificed mice had their tracheas surgically exposed and cannulated with 27-gauge silicon tubing attached to a 23-gauge needle on a 1-ml tuberculin syringe. Following instillation of 600 μl of sterile saline through the trachea into the lung, BALF was withdrawn and cytospun (3 min at 500 rpm) onto microscope slides. Eosinophil counts were performed as described above.

Peripheral blood. Blood was collected from the carotid artery. RBC were lysed using a 1:10 solution of 100 mM potassium carbonate, 1.5 M ammonium chloride. The remaining cells were cytospun (3 min at 500 rpm) onto microscope slides and air dried. Eosinophil counts were performed as described above.

Bone marrow. Bone marrow cells were flushed from femurs with 1 ml PBS and cytospun onto microscope slides, and separate slides were stained with Wright-Giemsa and DAB for cell differential counts.

Stimulation of splenocytes in vitro by ISS-ODN and anti-CD3 Abs

Ninety-six-well flat microtiter plates were coated with rat anti-mouse anti-CD3 Abs (1 μg/ml; PharMingen, San Diego, CA) at room temperature for 2 h and then washed with PBS. Splenocytes (5×10^6/ml) from each mouse (female BALB/c, $n = 4$) were incubated with ISS-ODN or M-ODN (10 μg/ml) at 37°C for 2 h and then added to anti-CD3-coated plates (5×10^5 cells/well), in triplicate, with or without neutralizing Abs to mouse IL-12, IFN-γ, and IFN-α (Biosource, Camarillo, CA). The optimal concentration of each neutralizing Ab used was determined previously in pilot experiments performed to neutralize the levels of IL-12, IFN-γ, and IFN-α produced by ISS-ODN-stimulated splenocytes (data not shown). Splenocyte supernatants (24, 48, and 72 h poststimulation) were assayed in duplicate to determine the level of each cytokine. All cytokines were analyzed by ELISA.

Administration of ISS to OVA-sensitized, but not OVA inhalation-challenged mice in vivo

Female BALB/c mice were sensitized to develop a Th2 response to OVA, as detailed above and in Fig. 2, but without subsequent aerosol OVA inhalation challenge. Eight weeks after the last OVA/alum injection, mice were injected i.p. with 100 μg of ODN, 1 day (−1d) or 3 days (−3d) before their sacrifice. The cytokine profiles of the supernatants derived from splenocytes incubated for 72 h with 100 μg/μl of OVA were assayed by ELISA.

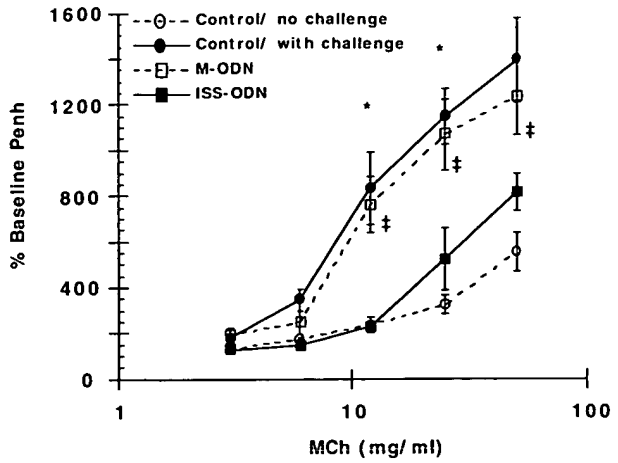

FIGURE 1. Administration of ISS inhibits MCh airway responsiveness. To determine the effect of ISS on airway responsiveness in OVA-sensitized mice, 50 μg of either ISS-ODN (n = 12 mice) or M-ODN (n = 12 mice) was injected i.p. 24 h before each of three OVA inhalation challenges, and airway responsiveness was determined 48 h after the last OVA challenge (5 days after the first dose of ISS). Airway responsiveness to increasing concentrations of nebulized MCh (0–50 mg/ml) was assessed by barometric whole body plethysmography, and Penh values were calculated. Means ± SEM of Penh values from three independent experiments are expressed as the percentage of baseline Penh values observed after PBS exposure. Administration of ISS-ODN significantly reduced the increase in MCh airway responsiveness by 69 ± 9.9% compared with M-ODN-treated mice (p = < 0.05).

Cytokine assays

The levels of various cytokines (IL-3, IL-5, GM-CSF, and IFN-γ) were measured in cell supernatants following either anti-CD3 Ab or OVA Ag stimulation by ELISA (PharMingen), as was previously described (10, 11, 16).

Eosinophil apoptosis assay

Eosinophils of >90% purity and >95% viability were purified from the blood of IL-5 transgenic mice using a Percoll gradient, as previously described in this laboratory (17). The eosinophils were then treated with ISS-ODN (1 μg/ml), M-ODN (1 μg/ml), or controls, including mouse rGM-CSF (1 ng/ml) (PharMingen) and anti-Fas Ab (1 μg/ml) (clone Jo2 from PharMingen), and analyzed at 2, 8, 18, and 32 h after treatment. Eosinophil apoptosis was measured by quantitating the number of apoptotic nuclei relative to healthy nuclei by cell permeabilization, propidium iodide staining, and FACS analysis, as described (18).

Statistical analysis

Statistical analysis was performed with ANOVA and Student's t test, as previously described (10–11). In studies of airway responsiveness, groups were compared by Tukey-Kramer HSD test. A p value of <0.05 was considered statistically significant.

Results

ISS-ODN inhibits allergen-induced airway hyperresponsiveness

Airway responsiveness to MCh was increased significantly in mice following OVA sensitization and OVA inhalation challenge, as opposed to mice sensitized to OVA alone with PBS challenge (14 ± 1.8-fold increase in Penh values OVA versus PBS following inhalation of 50 mg/ml MCh). Mice sensitized to OVA without inhalation challenge, or mice OVA challenged without OVA sensitization showed minimal change in Penh in response to MCh (data not shown).

Administration of ISS-ODN i.p. before each inhalation challenge significantly reduced the increase in MCh airway responsiveness in OVA-sensitized and OVA-challenged mice by 69 ± 9.9% (Fig. 1). These changes in airway responsiveness induced by ISS-ODN were accompanied by a significant reduction in BALF eosinophilia (M-ODN-treated mice, 253 ± 207 × 10^3 BALF eosinophils versus ISS-ODN-treated mice, 14 ± 12 × 10^3 BALF eosinophils) ($p < 0.05$).

Kinetics of ISS-ODN-mediated inhibition of airway and lung eosinophilia

To investigate the mechanism by which ISS inhibits airway eosinophilia, the number and route of OVA injections and inhalations in the mouse protocol were modified (depicted in Fig. 2). Both OVA protocols induce significant BAL eosinophilia (47 ± 16% BAL eosinophils with protocol used to assess airway responsiveness versus 42 ± 4% BAL eosinophils with protocol depicted in Fig. 2). However, the OVA protocol depicted in Fig. 2 induces an approximate eightfold greater absolute number of BAL eosinophils/ml compared with the protocol used to assess airway hyperresponsiveness (2075 × 10^3 BAL eosinophils versus 253 × 10^3 BAL eosinophils). Using this protocol (Fig. 2), OVA-sensitized and OVA inhalation-challenged mice developed significant airway eosinophilia (Fig. 3) compared with control PBS-challenged mice in the BALF (42 ± 4% versus 0% BALF eosinophils) and lungs (67 ± 5 eosinophils/hpf versus 2 ± 1 eosinophils/hpf, $p < 0.05$, Fig. 4). Even with the stronger stimulus for eosinophil recruitment in this protocol, ISS-ODN significantly inhibited eosinophil recruitment to BALF (91% inhibition compared with M-ODN) (Fig. 4*A*) and lung tissue (90% inhibition compared with M-ODN) (Fig.

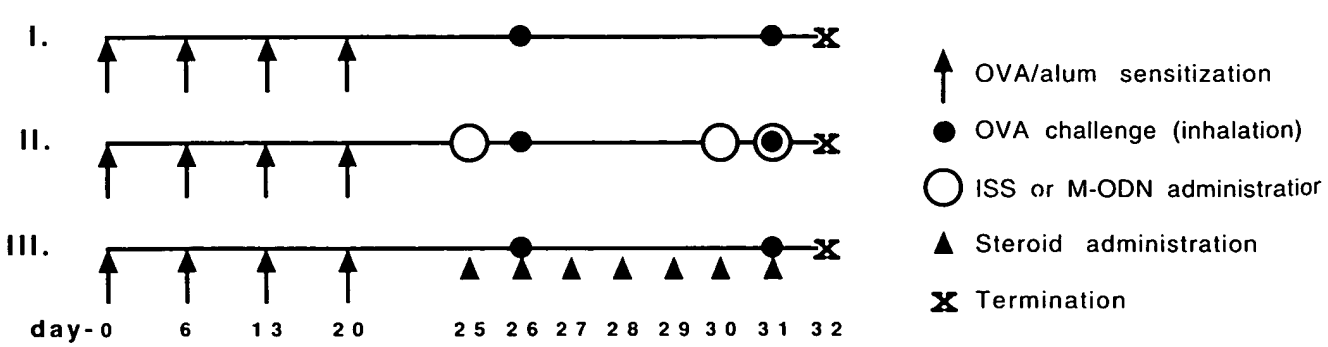

FIGURE 2. Protocol for induction of pulmonary allergic inflammation. *I*, Basic protocol. *II*, Therapeutic intervention with ISS-ODN or M-ODN. *III*, Intervention with corticosteroids. For details, see *Materials and Methods*.

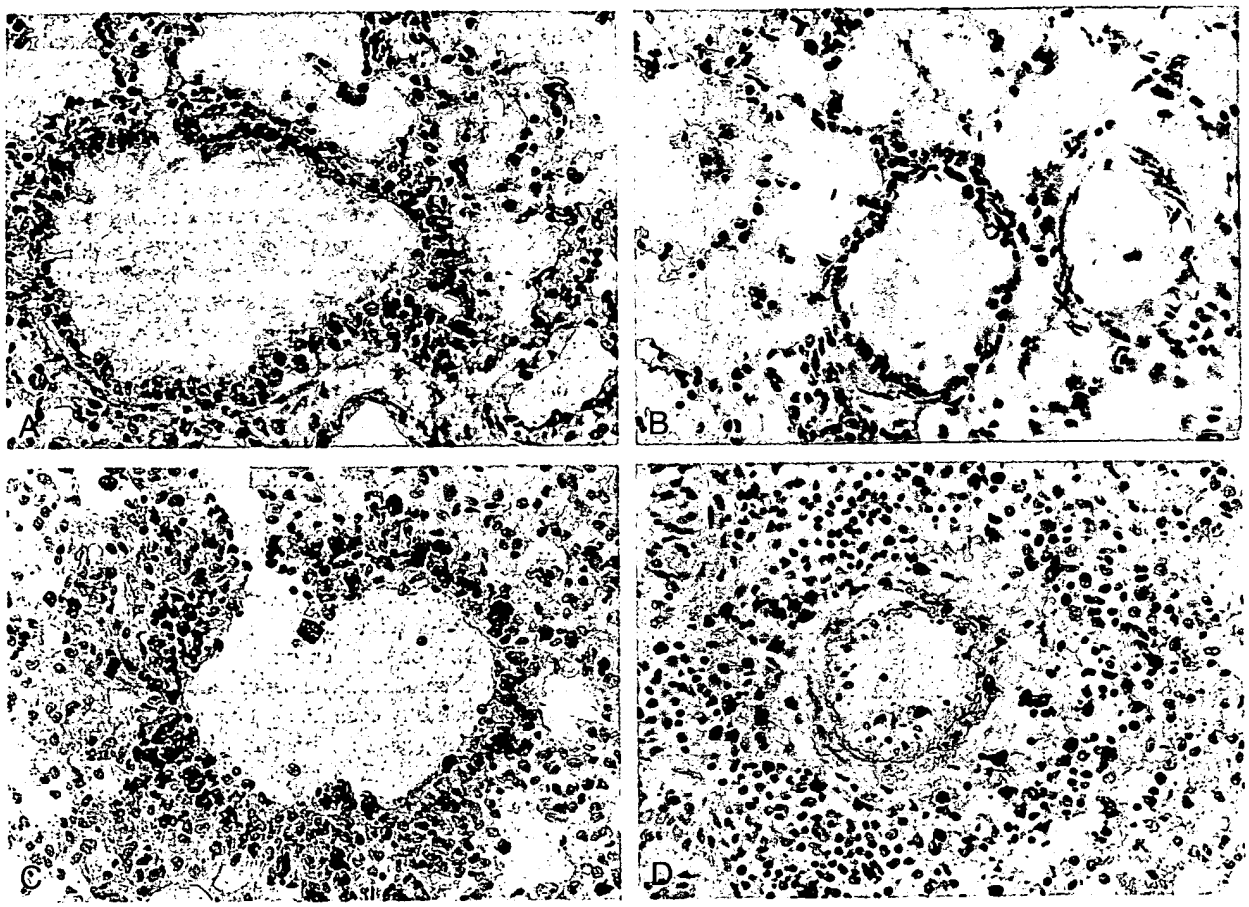

FIGURE 3. Eosinophilic infiltration in the lungs of mice treated with ISS-ODN or M-ODN OVA/alum-sensitized and OVA inhalation-challenged mice were treated i.p. with either ISS-ODN or M-ODN 6 days before the end of the experiment. Lungs were cryosectioned and stained to detect eosinophil peroxidase using the peroxidase substrate DAB. Cells expressing eosinophil peroxidase develop a brown color reaction. Peribronchial pulmonary eosinophilia was inhibited significantly in OVA-sensitized and OVA-challenged ISS-ODN-treated mice (*A*) compared with M-ODN-treated mice (*C*) (magnification ×40). Similarly, pulmonary lung eosinophilia was inhibited significantly in ISS-ODN-treated mice (*C*) compared with M-ODN-treated mice (*D*). *C* and *D* (M-ODN treated) also display the inflammatory mononuclear cell infiltration pattern that is decreased in *A* and *B* (ISS-ODN treated). The pattern of inflammation observed in the lungs of the M-ODN-treated mice was similar to those obtained in control mice (see Fig. 4). The same method was applied for eosinophil detection in BALF, peripheral blood, and bone marrow (Figs. 4 and 6).

4*B*). ISS-ODN administered once 3 to 6 days before the termination of the experiment was the optimal time point (of those examined) for ISS administration in inhibiting eosinophil recruitment into BAL and lung (Fig. 4, and data not shown). These studies demonstrate the sustained 6-day inhibitory effect of a single dose of ISS on subsequent allergen-induced eosinophil recruitment.

We also examined the time of onset of action of a single dose of ISS in inhibiting eosinophil recruitment. These studies demonstrated that a single dose of ISS had an initial onset of action in inhibiting eosinophil recruitment when administered as late as 1 day before termination of the experiment (ISS coadministered with OVA) (43% inhibition of BALF eosinophlia and 69% inhibition of lung tissue eosinophilia). This inhibitory effect was more prominent when ISS was administered 2 days before termination of the experiment (76% inhibition of BALF eosinophilia and 75% inhibition of lung tissue eosinophilia). Overall, these studies suggest that ISS-ODN inhibits airway eosinophilia by mechanisms that are both rapid in onset (onset of action within 24 h of ISS administration) as well as sustained (inhibitory effect sustained for at least 6 days after ISS administration).

ISS did not affect the number of BAL neutrophils, lymphocytes, or mononuclear cells. However, as there is not a significant increase in these cell types in BAL in models of allergen-induced airway inflammation, this may not be the appropriate model to determine whether ISS effects these cell types.

Mucosal administration of ISS-ODN inhibits airway and lung eosinophilia

Not only systemic (i.p.) ISS-ODN administration, but also mucosal (i.e., i.n. or i.t.). ISS-ODN administration had a similar inhibitory effect on BALF and lung eosinophil accumulation (Fig. 6, *A* and *B*).

Effect of ISS on peripheral blood and bone marrow eosinophil numbers and eosinophil apoptosis

ISS not only inhibited eosinophilia in the airway (by 91%) and lung parenchyma (90%), but also inhibited blood eosinophilia (86%) (Fig. 4*C*), suggesting that ISS was exerting a significant effect on the bone marrow production of eosinophils (number of bone marrow eosinophils inhibited 58%) (Fig. 4*D*). OVA challenge increased the total number of nucleated cells in the bone marrow of M-ODN-treated mice ($19.8 \pm 9.2 \times 10^6$ cells/ml) ($p = 0.04$ versus ISS-ODN), whereas ISS-ODN treatment of OVA-challenged mice ($7.5 \pm 1.8 \times 10^6$ cells/ml) reduced the total number of bone marrow cells to a level similar to that noted in naive mice not treated with ODN ($4 \pm 0.3 \times 10^6$ cells/ml). ISS inhibited the total number of bone marrow eosinophils (M-ODN, 1812 ±

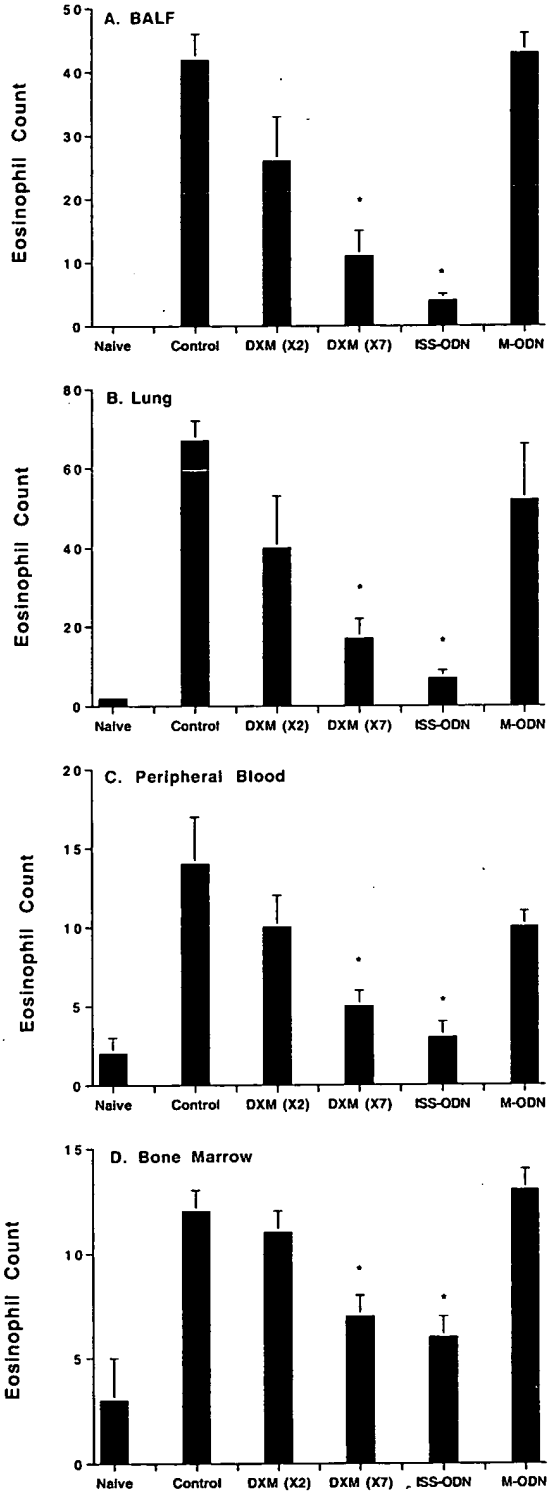

FIGURE 4. The effect of systemic ISS or corticosteroids on BALF, lung, blood, and bone marrow eosinophil levels. Groups of mice were sensitized and challenged with OVA, or OVA sensitized and pretreated i.p. with either ISS-ODN, M-ODN, or corticosteroids (2 daily doses or 7 daily doses) starting before the first OVA inhalation challenge. The number of eosinophils in BALF (*A*), lung (*B*), blood (*C*), and bone marrow (*D*) were quantitated with DAB staining (magnification ×40). Results are expressed as the mean ± SE (n = 8 per group) and, for BALF, peripheral blood, and bone marrow, represent eosinophils as a percentage of total nucleated cells. The results for lung (#) represent the number of eosinophils per microscopic field. The ODN dose was 100 μg/administration. The steroid dexamethasone (DXM) dose was 5 mg/kg. See Fig. 2 for details of the timing of intervention, sensitization, and challenge. PB, peripheral blood; BM, bone marrow. *, Denotes $p < 0.05$ versus the control group.

614×10^3 bone marrow eosinophils/ml versus ISS, $349 \pm 101 \times 10^3$ bone marrow eosinophils/ml, $p = 0.05$) and reduced the absolute number of peripheral blood eosinophils (M-ODN, $2815 \pm 995 \times 10^3$ eosinophils/ml versus ISS, $1390 \pm 44 \times 10^3$ eosinophils/ml, $p = 0.05$), but did not affect the total peripheral blood white blood cell count (M-ODN, $2.3 \pm 0.4 \times 10^4$ white blood cells/μl versus ISS, $2.6 \pm 0.3 \times 10^4$ white blood cells/μl).

The effect of ISS on reducing the number of eosinophils was not due to ISS directly inducing eosinophil apoptosis, as assessed in eosinophil apoptosis experiments in vitro. The percentage of apoptotic eosinophils after incubation for 18 h with ISS-ODN (51%) did not differ significantly from the percentage of apoptotic eosinophils after incubation with M-ODN (54%), or from the percentage of apoptotic eosinophils in untreated control cultures (55%). In contrast, positive and negative control experiments demonstrated that anti-Fas induced a significant degree of eosinophil apoptosis (87%), whereas GM-CSF protected eosinophils from apoptosis (5% apoptotic eosinophils).

Effect of ISS on the generation of IL-5

To investigate potential immunomodulatory mechanisms responsible for the ISS-induced inhibition of airway eosinophilia, the effect of ISS-ODN on IL-5 generation was evaluated. IL-5 induces eosinophil proliferation, differentiation, and resistance to apoptosis (1, 4), and genetically engineered elimination of IL-5 in mice in vivo dramatically reduces eosinophil numbers, as shown by the lack of eosinophils in IL-5 knockout mice (19). We evaluated whether inhibition of IL-5 production was responsible for ISS-ODN-mediated inhibition of bone marrow, peripheral blood, and airway eosinophilia. Systemic (i.p.) administration of ISS-ODN to OVA-sensitized and OVA-challenged mice reduced IL-5 production by 84%, while simultaneously inducing a 30-fold increase in IFN-γ production by OVA-stimulated $CD4^+$ splenocytes (Fig. 5). The kinetics of ISS-induced inhibition of IL-5 correlated with the inhibitory effect of ISS on airway eosinophilia in vivo. Similar results, i.e., suppression of IL-5 and induction of IFN-γ by $CD4^+$ splenocytes, were observed with mucosal ISS-ODN administration (i.n. and i.t.) (Fig. 7).

ISS inhibition of the generation of the eosinophil active cytokines (IL-5, GM-CSF, IL-3) is partially mediated by IL-12 and IFNs

We also monitored the effects of ISS-ODN on OVA/alum-sensitized, but nonchallenged mice. These OVA-sensitized mice provide an in vivo model to study whether ISS-ODN can inhibit the ability of an atopic mouse to release eosinophil active cytokines (IL-5, GM-CSF, IL-3). We postulated that administration of ISS-ODN alone (in the absence of Ag challenge) would stimulate the in vivo release of innate cytokines (IFN-α,β, IFN-γ, and IL-12), and that these cytokines would inhibit the secretion by T cells of eosinophil active cytokines (IL-5, GM-CSF, IL-3) without the induction of a subsequent Ag-specific Th1 response (no OVA Ag was administered by inhalation). In these studies, we evaluated OVA-stimulated spleen cell (derived from ISS-ODN-treated OVA-sensitized mice) production of IL-5, IL-3 (Table I), and GM-CSF (Table II), all of which are similar in their ability to induce eosinophil proliferation, and block eosinophil apoptosis through their activation of a common β-chain shared by these cytokine receptors (4). As shown in Table I, i.p. injection of ISS-ODN (1 day or 3 days before the in vitro cytokine assay) reduced secretion of IL-5 (83% inhibition group C versus A) and IL-3 (76% inhibition, group C versus A) by OVA-stimulated splenocytes without inducing any OVA-specific IFN-γ production. The experiments in Table I (OVA sensitization with no OVA challenge) and Fig. 5

Table I. *In vivo ISS-ODN administration of OVA-sensitized mice inhibits IL-5 without induction of IFN-γ*[a]

Group	Treatment	IL-3 (pg/ml)	IL-5 (pg/ml)	IFN-γ (pg/ml)
A	Control	1299 ± 89	657 ± 52	< 20
B	ISS-ODN (-1d)	309 ± 26[b]	112 ± 18[b]	< 20
C	ISS-ODN (-3d)	463 ± 48[b]	144 ± 27[b]	< 20
D	M-ODN (-1d)	964 ± 81	508 ± 77	< 20

[a] Female BALB/c mice were sensitized to develop a Th2 response to OVA as detailed in Fig. 2, but without subsequent aerosol OVA challenge. Eight weeks after the last OVA/alum injection, mice were injected i.p. with 100 μg of ODN, 1 day (-1d) or 3 days (-3d) prior to their sacrifice. The cytokine profiles of the supernatants derived from splenocytes incubated for 72 h with 100 μg/ul of OVA were assayed by ELISA. Only baseline levels of IL-3, IL-5, and IFN-γ were detected in the supernatants of cultures incubated without OVA. No measureable levels of IL-4 and GM-CSF were detected in the various supernatants. Results are expressed as the mean ± SE (n = 4 for each group).

[b] Denotes $p < 0.05$, ISS-ODN-treated group vs control.

(OVA sensitization and OVA challenge) demonstrate that ISS can inhibit IL-5, GM-CSF, and IL-3 in OVA-sensitized mice in the presence or absence of OVA challenge. In contrast, ISS-treated mice require reexposure to OVA Ag to generate an OVA-specific Th1 response (induction of IFN-γ) (Figs. 5 and 7).

To address the role of innate cytokines (IL-12, IFNs) in mediating the in vivo inhibitory effects of ISS on the generation of IL-5, GM-CSF, and IL-3, we stimulated splenocytes in vitro with anti-CD3 Abs in the presence or absence of ISS-ODN and neutralizing Abs to IFN-α/β, IFN-γ, or IL-12 (Table III). In vitro incubation with ISS-ODN significantly inhibited anti-CD3-induced T cell production of IL-5 (47%), GM-CSF (49%), and IL-3 (47%) (Tables I and II). This inhibitory effect of ISS-ODN on eosinophil active cytokine release was partially mediated by the innate cytokines (IFNs and IL-12), as was shown in the related Ab neutralization studies (Table III). The in vitro stimulation of splenocytes with ISS-ODN and anti-CD3 Abs enhanced IFN-γ levels by threefold (data not shown). ISS stimulation neither inhibited IL-4 secretion nor increased IL-2 levels elicited by anti-CD3 stimulation (data not shown).

Investigations into the development of pulmonary eosinophilia and cytokine expression (IL-5 and IFN-γ) following a single ISS administration as compared with 7 days of corticosteroids

A single i.p. or i.n. ISS-ODN administration was as effective as seven daily doses of dexamethasone (5 mg/kg s.c.) in the suppression of airway eosinophilia (Figs. 4 and 6). A single dose of dexamethasone was not effective in inhibiting eosinophilic airway inflammation (data not shown), whereas two doses of dexamethasone had a partial effect on inhibiting eosinophil recruitment into the lung (Figs. 4 and 5). While both ISS and dexamethasone inhibited IL-5 generation (Figs. 5 and 7), only ISS was able to induce IFN-γ (Figs. 5 and 7).

Table II. *Effect of in vitro ISS-ODN stimulation on anti-CD3-induced T cell cytokine production*[a]

Stimulation	IL-3 (pg/ml)	IL-5 (pg/ml)	GM-CSF (pg/ml)
Anti-CD3	2495 ± 121	1553 ± 120	1090 ± 141
Anti-CD3/ISS-ODN	1183 ± 217[b]	730 ± 230[b]	535 ± 118[b]
Anti-CD3/M-ODN	4975 ± 848	1795 ± 116	1951 ± 538

[a] Splenocytes (5 × 10[6]/ml) were incubated with ISS-ODN or M-ODN (10 μg/ml) at 37°C for 2 h and then added to anti-CD3-coated plates (5 × 10[5] cells/well) in triplicate. The cytokine profiles of the supernatants were assayed by ELISA. Results are expressed as the mean ± SE (n = 4 per group).

[b] Denotes $p < 0.05$, anti-CD3 plus ISS-ODN-treated group vs. anti-CD3-treated group.

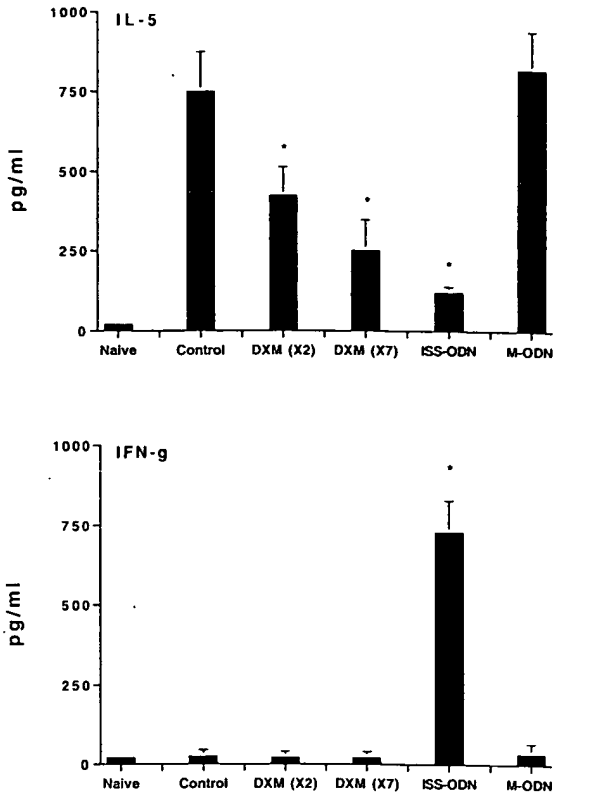

FIGURE 5. The effect of systemic ISS or corticosteroids on IL-5 and IFN-γ levels. Groups of mice were sensitized and challenged with OVA, or OVA sensitized and pretreated i.p. with either ISS-ODN, M-ODN, or corticosteroids (2 daily doses or 7 daily doses) starting before the first OVA inhalation challenge. The cytokine profiles of $CD4^+$ splenocyte supernatants were assayed by ELISA from cells incubated for 72 h with 100 μg/μl of OVA. Only baseline levels of IL-5 and IFN-γ were detected in the supernatants of cultures incubated without OVA. Results are mean ± SE (n = 8 per group).

Discussion

In this study, we demonstrate that ISS inhibits airway eosinophilia and prevents the development of airway hyperresponsiveness. These observations are similar to those noted by Kline et al. (20) in a different mouse model of airway inflammation. In the present study, we have extended these observations by demonstrating 1) the novel mechanism by which ISS inhibits airway eosinophilia

Table III. *In vitro ISS-ODN stimulation of anti-CD3-induced T cell cytokine production: effect of neutralizing Abs to innate cytokines (IL-12 and IFNs)*[a]

Stimulation	IL-3 (pg/ml)	IL-5 (pg/ml)	GM-CSF (pg/ml)
Anti-CD3/ISS-ODN	1183 ± 217	730 ± 230	535 ± 118
Anti-CD3/ISS-ODN and anti-IL-12	1154 ± 209	870 ± 263	829 ± 153[b]
Anti-CD3/ISS-ODN and anti-IFN-γ	1169 ± 214	2218 ± 231[b]	840 ± 222[b]
Anti-CD3/ISS-ODN and anti-IFN-α/β	1565 ± 228[b]	753 ± 215	682 ± 122[b]

[a] Splenocytes (5 × 10[6]/ml) were incubated with ISS-ODN or M-ODN (10 μg/ml) at 37°C for 2 h and then added to anti-CD3-coated plates (5 × 10[5] cells/well) in triplicate, with or without neutralizing Abs to mouse IL-12, IFN-γ, and IFN-α (Biosource). The cytokine profiles of the supernatants were assayed by ELISA. Results are expressed as the mean ± SE (n = 4 per group).

[b] Denotes $p < 0.05$, anti-CD3 plus ISS-ODN treated group vs anti-CD3 plus ISS-ODN plus cytokine neutralizing Ab-treated group.

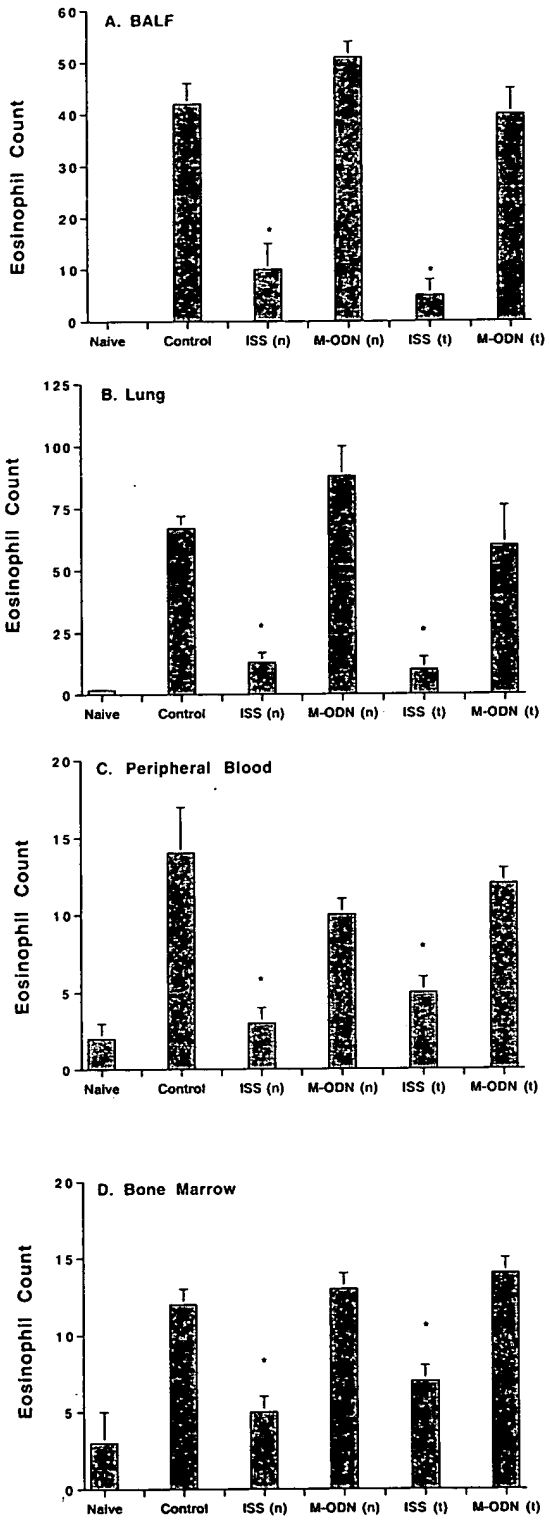

FIGURE 6. The effect of mucosal administration of ISS on BALF, lung, blood, and bone marrow eosinophil levels. Groups of mice were sensitized and challenged with OVA, or OVA sensitized and pretreated i.n. (n) or i.t. (t) with either ISS-ODN or M-ODN, starting before the first OVA inhalation challenge. The number of eosinophils in BALF (*A*), lung (*B*), blood (*C*), and bone marrow (*D*) were quantitated with DAB staining. Results are expressed as the mean ± SE (n = 8 per group) and, for BALF, peripheral blood, and bone marrow, represent eosinophils as a percentage of total nucleated cells. The results for lung (#) represent the number of eosinophils per microscopic field. The ODN dose was 100 μg/administration. See Fig. 2 for details of the timing of intervention, sensitization, and challenge. PB, peripheral blood; BM, bone marrow; n, intranasal, t, intratracheal. *, Denotes $p < 0.05$ versus the control group.

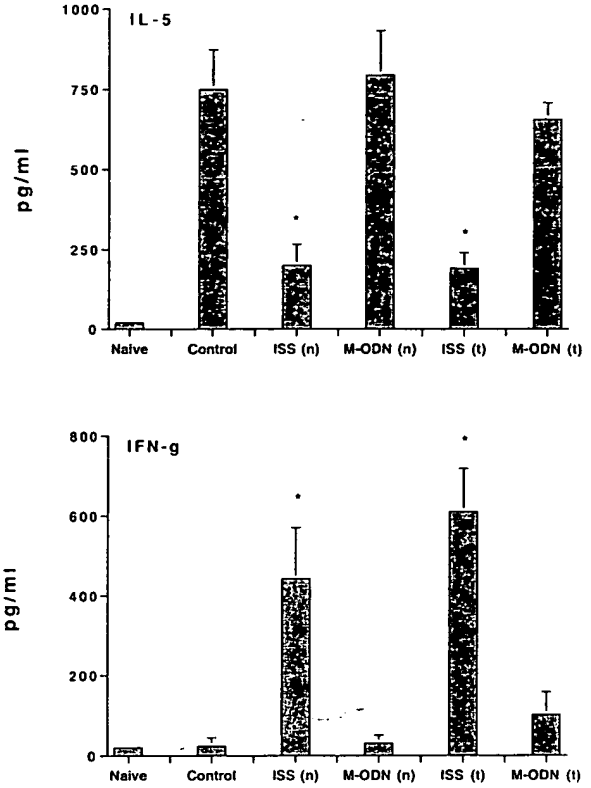

FIGURE 7. The effect of mucosal administration of ISS on IL-5 and IFN-γ levels. Groups of mice were OVA sensitized and OVA challenged, or OVA sensitized and pretreated i.n. (n) or i.t. (t) with either ISS-ODN or M-ODN, starting before the first OVA inhalation challenge. The cytokine profiles of $CD4^+$ splenocytes were assayed by ELISA utilizing supernatants from cells incubated for 72 h with 100 μg/μl of OVA. Only baseline levels of IL-5 and IFN-γ were detected in the supernatants of cultures incubated without OVA. Results are mean ± SE (n = 8 per group). n, intranasal; t, intratracheal challenge. *, Denotes $p < 0.05$ versus the control group.

through inhibition of the production and release of eosinophils from the bone marrow; 2) that inhibition of bone marrow production of eosinophils was associated with a significant inhibition of IL-5, GM-CSF, and IL-3 production; 3) that ISS exerted this inhibitory effect on T cell cytokine production indirectly by stimulating monocytes/macrophages and NK cells to generate IL-12 and IFNs, as demonstrated in in vitro neutralizing Ab studies; 4) that the effect of ISS on reducing the number of tissue eosinophils was both immediate (onset within 1 day) and sustained (over 6 days), and was not due to ISS directly inducing eosinophil apoptosis; 5) that ISS was effective in inhibiting eosinophilic airway inflammation when administered either systemically or mucosally (i.e., i.n. or i.t); 6) that a single administration of ISS (systemic or mucosal) inhibited airway eosinophilia as effectively as daily systemic administrations of corticosteroids for 7 days; and 7) that while both ISS and corticosteroids inhibited IL-5 generation, only ISS was able to induce IFN-γ (a cytokine that importantly biases the immune system to generate a Th1 and not a Th2 response to subsequently encountered allergens). Thus, systemic or mucosal administration of ISS before allergen exposure provides a novel form of active immunotherapy in allergic diseases.

The inhibitory effects of ISS are likely to be mediated by the innate cytokines derived from monocytes/macrophages and NK cells stimulated by ISS (6, 9, 10). In studies utilizing purified populations of mouse bone marrow-derived macrophages, we have

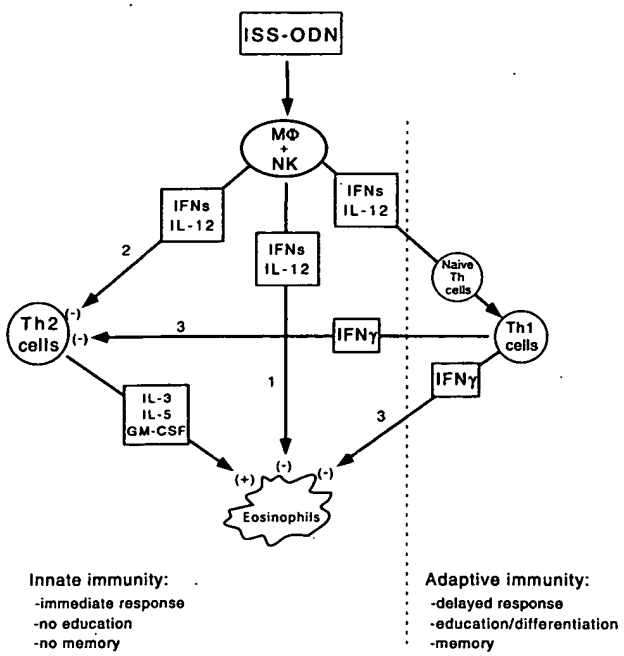

FIGURE 8. Mechanisms by which ISS-ODN suppress eosinophil accumulation in the lungs. ISS-ODN activates the innate immune system (macrophages and NK cells) to release IFNs (α, β, γ) and IL-12. These cytokines inhibit eosinophil recruitment and activation (step 1), as described (23–25). In addition, as shown in this study, these cytokines down-regulate the secretion by Th2 cells of IL-5, IL-3, and GM-CSF, cytokines that induce eosinophil proliferation in the bone marrow (step 2). The IFNs and IL-12 bias naive Th cells toward allergen-specific Th1 differentiation. Once generated, the Th1 cells secrete IFN-γ in response to allergen, which inhibit further Th2 activation and differentiation as well as eosinophil recruitment and activation (step 3) (26). Steps 1 and 2 occur within hours of exposure to ISS-ODN and are mediated by the innate immune system, while step 3 is the product of adaptive immunity. Furthermore, while steps 1 and 2 have transient antieosinophilic effects, step 3 leads to immunologic memory. Once established, allergen-specific Th1 responses can efficiently inhibit eosinophilic inflammation upon subsequent allergen exposure (step 3).

demonstrated recently that ISS induces these macrophages to release IL-12, but not IFN-γ.[4] In additional studies,[4] stimulation of cultured splenocytes derived from SCID mice with ISS induced the release of both IL-12 and IFN-γ. As purified populations of BALB/c-derived macrophages stimulated with ISS did not generate IFN-γ,[4] these studies with SCID mice suggest that NK cells may be the predominant source of IFN-γ in SCID mice stimulated with ISS. These[4] and other studies (10, 21) suggest that ISS is able to stimulate both macrophages and NK cells to produce cytokines that modulate T cell cytokine production.

ISS-ODN administration, via activation of innate immunity, could inhibit pulmonary eosinophilia through at least three different, but additive mechanisms (Fig. 8). The first mechanism by which ISS can inhibit pulmonary eosinophilia is through an effect on T cell-derived cytokines important to the bone marrow generation of eosinophils. ISS exerts this inhibitory effect indirectly by stimulating monocytes/macrophages and NK cells to generate IL-12 and IFNs that subsequently inhibit T cell generation of IL-5, GM-CSF, and IL-3. The greater inhibition by ISS of peripheral blood eosinophilia (86% inhibition) compared with inhibition of bone marrow eosinophilia (58% inhibition) suggests that ISS may have also inhibited release of eosinophils from the bone marrow. In this regard, IL-5 is known to induce release of eosinophils from the bone marrow (22), and inhibition of IL-5 generation by ISS could thus prevent bone marrow release of eosinophils. A second mechanism by which ISS-induced generation of IFNs and IL-12 could inhibit pulmonary eosinophilia is through an effect on eosinophil recruitment, as has previously been demonstrated with IL-12 (23, 24), IFN-α (25), and IFN-γ (26) in models of allergic inflammation and parasitic infection. A third eosinophil-inhibitory mechanism induced by ISS is the generation of an allergen-specific Th1 as opposed to a Th2 response. This would be important for long-term protection and immunologic memory. This ISS-induced OVA-specific Th1 response would generate IFN-γ, which further inhibits eosinophil accumulation by biasing naive T cells encountering Ag in an IFN-γ milieu to generate Th1 as opposed to Th2 responses to newly encountered allergens. The first two inhibitory mechanisms affecting pulmonary eosinophilia are most likely mediated by the innate immune response, and are therefore primarily immediate and Ag nonspecific in nature. In contrast, the third effect is mediated by an adaptive immune response and requires a longer period of time for differentiation and maturation of Ag-specific Th1 cells from naive $CD4^+$ T cells (Fig. 8). Furthermore, while the first two mechanisms lead to a dramatic, but probably temporary reduction in eosinophil recruitment, the third mechanism is involved in the generation of immunologic memory that may prevent Th2 cell responses and eosinophil recruitment into the target organ (i.e., the lung) from developing following subsequent airway allergen challenge.

The effect of ISS on reducing the number of tissue eosinophils was both immediate (onset within 1 day) and sustained (over 6 days), and was not due to ISS directly inducing eosinophil apoptosis. Administration of ISS-ODN 6 days before the final OVA inhalation challenge was more effective in inhibiting pulmonary eosinophil infiltration than simultaneous delivery of ISS-ODN and OVA 1 day before the end of the experiment. Administration of ISS-ODN 6 days before the final OVA inhalation challenge also generated OVA-specific Th1 responses (induction of IFN-γ) and attenuated preexisting OVA-specific Th2 responses (i.e., reduction of IL-5, Figs. 5 and 7). The administration of ISS 1 day before, or together with the final OVA inhalation challenge was sufficient to inhibit pulmonary eosinophil recruitment as well as the generation of eosinophil active cytokines 24 h later. However, this method of ISS administration did not result in OVA-specific Th1 responses, probably because Ag-specific IFN-γ production generally requires more than 24–48 h.

The activation of the innate immune response by ISS-ODN could initially prevent the development of asthmatic symptoms by preventing early eosinophil accumulation, and later even bias the immune system to generate an allergen-specific Th1 response to any allergen subsequently encountered by the host in his own natural environment (Fig. 8). Thus, therapy with ISS has potential beneficial immunomodulatory effects on allergic inflammation not noted with corticosteroids. For example, therapy with corticosteroids, while effective in inhibiting IL-5 generation, did not induce IFN-γ (Fig. 5). The potential benefit of ISS therapy in comparison with corticosteroids in allergic disease would be the ability of ISS to alter the cytokine mileu of the host (IFN-γ) to favor generation of Th1 responses to subsequently encountered allergens.

A recent study demonstrated an inverse epidemiologic association between exposure to mycobacteria and the prevalence of atopic disorders, suggesting that the relatively recent decline in infections (e.g., tuberculosis) in developed countries is a factor underlying the increasing severity and prevalence of allergic diseases (27). Furthermore, infection of mice with *Mycobacterium*

[4] E. Martin-Orozco, H. Kobayashi, M.-D. Nguyen, J. Van Uden, R. S. Kornbluth, and E. Raz. Activation of APCs by immunostimulatory DNA sequences. *Submitted for publication.*

bovis Bacillus Calmette Guerin has been shown to suppress allergen-induced airway eosinophilia (28). As previously mentioned, the ISS were initially identified and isolated from mycobacterial DNA (5, 6) and they appear at the expected frequency in many pathogenic bacteria and viruses, while they are underrepresented in the vertebrate genome (7). Thus, the natural exposure to ISS-enriched DNA from normal intracellular pathogens, through infection, may play a role in shaping the immune response away from an allergic phenotype and allergic responses following allergen challenge. As shown in this study, ISS-ODN can provide this immunomodulatory effect without the risk of inducing active infection.

In summary, ISS-ODN administration provides an alternative to the current practice of allergen protein desensitization, which has relatively low efficacy and high potential for significant side effects, including anaphylaxis (29, 30). ISS-ODN delivery via the systemic or mucosal route (i.n. or i.t.) in allergic patients may inhibit the allergic inflammatory responses in asthmatic lungs after natural allergen exposure. The future application of ISS-ODN therapy to human allergic diseases will depend on how well the inhibitory effects of ISS-ODN noted in murine models of experimental allergic airway inflammation translate to related human diseases.

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